Chapter 8
Single-Pollen Genotyping of Holocene Lake Sediments

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8.1 Ancient DNA Analysis

Genetic analyses of fossilized materials are now possible by means of new techniques such as polymerase chain reaction (PCR) and the more recent next generation sequencing techniques. These techniques have attracted much attention in all areas of biology during the past two decades. By directly analyzing DNA from fossil samples of different ages collected from different sites, it is now possible to study the history of ancient taxa, clarify the migration routes followed after glaciation, and understand the microevolutionary processes that occurred in the recent past. In the past two decades, ancient DNA (aDNA) has been successfully extracted from fossilized organisms, and results from specimens of late Quaternary remains (up to 100 thousands of years, ka) have provided insights into many evolutionary processes, particularly in animal species (Krings et al. 1997; Cooper et al. 2001; Lambert et al. 2002; Shapiro et al. 2004; Gilbert et al. 2008). In addition, the combination of coalescent methods (Hein et al. 2005) and aDNA sequences is a very powerful tool to reconstruct detailed demographic histories, test models of population genetics, and understand the interplay between microevolution and environment (Hadly et al. 2004; Ramakrishnan and Hadly 2009). In plants, unfortunately, methodological difficulties and the rarity of suitable well-preserved samples have often prevented the widespread application of aDNA studies, especially at the population level, because well-preserved hard tissues such as wood are difficult to obtain in sufficient numbers over sufficiently large geographic areas.

8.2 Why Fossil Pollen?

Fossilized pollen grains of vascular plants are the most abundant and among the best preserved remains of many groups of plants (Faegri and Iversen 1989). During the past 50 years, fossil pollen have been the key tools used by paleoecologists for elucidating the environmental conditions during the previous thousands of years. The sediments of lakes at highly elevated regions, such as in the mountains, contain pollen deposits from local plants; columnar sediment cores from the bottom of such lakes can be extracted (Fig. 8.1) and analyzed to reconstruct the structure of the land cover for thousands of years earlier. The cores of these sediments are often horizontally stratified because new sediments are regularly deposited into the lake each year. After establishing the age of a layer through radiocarbon dating, the abundance and type of pollen preserved within the sediment are analyzed. Routine analysis of the pollen usually reveals information about the genus or family, thereby providing a generalized view of the vegetation history of the region. Analysis of fossil pollen reveals that the distributions of species dispersal at the end of the last glaciation period were related to the modern patterns of genetic variation in many plant species, providing important linkages between temporal and modern patterns of variation in plants (e.g., in *Picea abies*; Tollefsrud et al. 2008).

Fig. 8.1 A sediment core sample (**c**) was collected (**a**) at Lake Holtjärnen (**b**)

Pollen retrieved from Holocene lake sediments from the cold regions of northern latitudes offers many advantages for aDNA studies. If the lake is confined to a specific area, favorable depositional conditions and fast burial rate reduce physical damage and exposure of the grains to biotic degradation by protecting the environment of the fossil and preventing percolation through the sediment sequence. Further, Holocene lake sediments contain high concentrations of pollen grains (>100,000 grains/cm^3), and as a result of the low temperatures these grains are more likely to have preserved DNA molecules because they are less affected by diagenesis. Another advantage is the relatively young age of pollens, which allows tracing of the lineages of Holocene specimens directly to the living taxa by using appropriate molecular markers. Thus, extant sequences can easily be compared with the ancient ones, and direct links can be established with modern populations. Finally, the fine degree of chronological precision that can be obtained from the Holocene specimens using radiometric dating methods enables the establishment of a detailed timescale using small amounts of material.

Pollen contains haploid DNA and is the means of its dispersal to the haploid DNA of the ovules. In flowering plants, the pollen disseminated from the flowers contains either two or three haploid cells. Both pollen types possess a large vegetative cell that encloses either a single or two generative cells. The vegetative cells comprise the bulk of pollen grain cytoplasm, including numerous plastids and mitochondria, and are responsible for the development of the pollen tube and delivery of the generative cells to the embryo sac together with the nuclear haploid DNA.

Therefore, multiple copies of organellar DNA (i.e., DNA of plastids and mitochondria) are present in the pollen of flowering plants. In addition, depending on the mode of inheritance of the organelles (paternal or maternal), there is a selective increase or decrease in the amount of organellar DNA in each pollen grain during pollen maturation (Nagata et al. 1999). Hence, pollen from species with paternal inheritance of plastid DNA, which include the majority of conifers, should be particularly rich in plastid DNA at maturation; that is, plastid DNA sequences are present in high copy number.

Because fossil DNA is the repository of the genetic history of an organism, this DNA is the direct consequence of evolution. In 1996, Suyama et al. succeeded in amplifying a short region of chloroplast DNA from the pollen grains of a 150,000-year-old *Abies* spp. plant collected from a Quaternary peat deposit at Kurota Lowland, Fukui, Japan. Some years later, by using the same technique we succeeded in isolating and analyzing short chloroplast DNA regions (<200 base pairs, bp) from Holocene pollen of Scots pine (*Pinus sylvestris* L.) retrieved from a postglacial lake sediment from central Sweden (Parducci et al. 2005). The method has also been recently applied to an angiosperm species (*Fagus sylvatica*; Paffetti et al. 2007) to analyze chloroplast variation in pollen grains as old as 45,000 years.

8.3 Pollen Treatment and DNA Extraction

The samples should be obtained from lake sediments with optimal and continuous preservation conditions (e.g., deep lakes from cold environments, which have a fast sedimentation rate). The pollen type should be as abundant as possible and easily distinguished from other pollen types present in the sediment. For example, the pollen grain of *P. sylvestris* has a 50-μm-long body with two laterally placed large bladders, and this pollen can be easily distinguished from that of *P. abies* with large body size, commonly longer than 75 μm, and a smooth transition between bladders and body.

Fieldwork, including drilling the core, and laboratory analyses must be performed during autumn and winter, to avoid the pollination period (i.e., spring), and specific methods should be used to avoid contamination (for details, see Parducci et al. 2005). We removed about 5 g wet weight of sediment samples from the core using a sterile scalpel in a DNA-free pollen laboratory that was located in a building physically separated from the one in which DNA analysis was performed. The upper 2-cm part from the surface of the sediment was previously discarded to avoid contamination during sampling. The samples were stored in sterile plastic bags in a freezer (−20°C). Subsequently, the sediment core was analyzed for pollen and macro remains, and then radiocarbon dating was performed. Before DNA analysis, about 0.5 g of each sample was dissolved in a few drops of sterile distilled water and the solution was sieved through filter cloths using distilled water. In the end, we collected approximately 2 ml of final solution containing the majority of the target pollen grains in a sterile petri dish and stored this solution at 5°C for no more than 2 days.

Next, the petri dishes were transferred to a second building in a pre-PCR lab equipped with a positive-pressure flow hood irradiated with ultraviolet radiation; 10–15 drops of soil solution was placed on glass slides and observed under a microscope with 10–40× magnification. Using standard micropipettes of 0.5–10 µl capacity, single pollen grains were selected under the microscope and transferred on clean glass slides. Each grain was washed 30–50 times with sterile distilled water drops, transferred to a clean glass slide, washed again 10–20 times, and finally moved to a sterilized PCR tube containing less than 0.5 µl sterilized water. For each sample, contamination by exogenous DNA was monitored by using a PCR blank that contained 1 µl solution from the last drop of water used for washing the grains.

For DNA extraction, we use a modified version of the extraction method described by Suyama et al. (1996) and Matsuki et al. (2007). To the PCR tube containing the pollen grain, we added 2 µl extraction buffer containing 0.01% sodium dodecyl sulfate (SDS), 0.1 g/l proteinase K, 0.01 M Tris–HCl (pH 7.8), and 0.01 M ethylenediaminetetraacetic acid (EDTA). The grain was crushed under the microscope using a sterile plastic pipette tip. The PCR tube was then closed and incubated at 37 or 56°C for 60 min and at 95°C for 10 min.

8.4 Choice of Markers and Amplification

PCR is a fundamental technique used in aDNA analysis. There are more chances of recovering aDNA from mitochondrial or chloroplast genomes of fossil pollen than from nuclear DNA because both mitochondria and chloroplasts are present in multiple copies in the vegetative cells of the grains and the plastid membranes afford extra protection to their DNA. In addition, the organellar DNA is conserved in its priming sites. The availability of a large database facilitates the designing of primers and selection of DNA regions with the appropriate level of polymorphism for testing of different hypotheses. However, because significant degradation of aDNA complicates the amplification of fragments longer than a few hundred base pairs, the amplification of target fragments longer than 200 bp is usually avoided.

Nevertheless, pollen grains are not a good source of aDNA because only one DNA region (nuclear, plastid, or mitochondrial) can be amplified from one pollen grain at one time; in contrast, several DNA regions can be analyzed at different times from DNA extracts obtained from multicellular samples (such as bone, tissue, or wood). This problem of pollen grains can be partially circumvented by analyzing several DNA regions in a single PCR reaction (multiplex PCR method) by using multiple primers (Chamberlain et al. 1988). In this way, the analysis of multiple regions can provide more detailed information on the nature of genetic change that occurred within individuals from different ages. At the same time, the amplification of regions from different genomes facilitates data validation in aDNA analysis. If more information can be simultaneously obtained from multiple assays, there is much less probability that the results are influenced by contamination. We performed multiplex PCR amplifications using a Multiplex PCR kit (Qiagen) with an

initial activation step at 95°C for 15 min, 35–40 cycles of denaturation at 94°C for 30 s, annealing at 55–60°C (depending on the primer pair mix) for 90 s, and extension at 72°C for 1 min, and a final step at 72°C for 10 min. The volume of the reaction mixture was 10 μl, including 2 μl extracted pollen DNA solution, 1× Multiplex PCR Master Mix (Qiagen), 0.2 μM each primer, and water (for adjusting the final volume). Using internal primers specific to the target sequence of interest, we performed a secondary amplification on the PCR products separated as single bands on 2% agarose gel. The 20-μl reaction mixture used for the latter procedure contained 1× HF PCR Buffer (Phusion; Finnzymes), 0.2 mM each dNTP, 0.5 μM each primer, and 0.02 unit/μl of Phusion DNA polymerase (Phusion; Finnzymes). The amplification conditions were as follows: initial activation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 10 s, annealing at 50–65°C for 10 s, and finally incubation at 72°C for 5 min. Secondary PCR products were purified, and 4–8 μl of this product was used for direct sequencing using internal primers. Each sequence was read from both strands. In addition, 3 μl of the purified secondary product was used for cloning, and a minimum of six clones per fragment were amplified, aligned, and their sequences compared with those obtained from direct dideoxy sequencing (forward and reverse sequences). Amplifications using fresh plant material were performed after those of aDNA.

The prerequisite for successful multiplex analysis is a good primer design that does not allow unspecific product generation, formation of extensive primer dimers, and preferential amplification that may result from unbalanced primer characteristics. In our case, we tried to design primers to obtain products that are as short as possible (<200 bp). In this type of analysis, DNA degradation is presumably the most common reason for PCR failure because most pollen grains do not contain significant amounts of intact targets more than 200 bp in length. The second important reason for amplification failure is the presence of inhibitors, and this can be easily identified by the lack of formation of primer dimer or the presence of any other unspecific amplification products on agarose gel. The inhibiting substances may come from soil, which contains components derived from decomposing plant material that inhibit the *Taq* polymerase activity.

8.5 Contamination

The studies on aDNA are complicated by several technical difficulties and require careful execution and practical expertise. Contamination is the most serious hazard for any researcher working with PCR techniques. The power and sensitivity of PCR, which generates large amounts of amplified product from as samples as small as less than a single cell, are the factors that increase the chances of contamination. Researchers working on modern DNA do not face this problem because their target DNA samples outnumber the other possible contaminating DNA molecules present in the PCR environment; this allows them to keep the number of PCR cycles as low as 25–30. However, the fewer numbers of cycles is not enough for the analysis of ancient sequences wherein the cycle numbers are usually up to 35–40.

Contamination may occur at different points during the analyses. First, it may occur during the handling of samples before PCR or while transferring the reagents during PCR. Second, contamination may occur within the PCR reaction tubes (contaminated reagents or disposable laboratory equipment purchased directly from the manufacturer). The latter is more difficult to avoid because it is beyond the laboratory's control, while contamination during handling can be detected by using an adequate set of control samples (blanks). Carryover contamination occurs when amplification products are introduced in the pre-PCR analysis step and is usually easy to detect. Such contamination can be disastrous and can be avoided by effectively separating the pre- and post-PCR areas, including all assigned equipment; irradiating workbenches and equipments with UV light; or simply by cleaning all surfaces with alkaline or acidic solutions (more environmentally friendly). On the other hand, cross-contamination or sample-to-sample contamination is usually more difficult to detect; however, this type of contamination is less frequent when handling pollen grains because no isolated DNA is involved in the pre-PCR steps. It requires, however, that all pre-PCR steps should be performed meticulously, such as carefully separating and handling grains, using effective cleaning techniques, changing disposable gloves on a regular basis, and using filter tips. In general, contamination that occurs because of personal handling of sediments, pollen grains, and reagents is detected by using sets of control samples consisting of negative controls; the approach to detect such contamination will vary with the mode of introduction of the contamination.

8.6 Validation of Results

To prove that only indigenous DNA and not the contaminating substance is amplified, it is necessary to repeat the entire experiment by using different DNA extracts (from the same sample) in a different laboratory (criterion of reproducibility). However, when pollen is the source of aDNA, only one type of DNA can be amplified from a pollen grain at one time. In such a case, reproducible results can be obtained by ensuring that a very large number of grains can be procured, thereby allowing statistical evaluation of the population genotypes, rather than giving primary importance to the successful extraction of genetic material from a few specimens (or even one specimen). In addition, when a very large number of samples can be obtained, the validation power of the reproducible results mainly depends on the particular information content of the obtained sequences. For example, the discriminating power of repeatedly obtained results is high when targeting variable regions, and comparable haplotypes can be obtained from the same population in two different laboratories. Hence, different results obtained from different individuals (grains) become a tool of self-validation if they can be obtained consistently (Montiel et al. 2001).

8.7 Conclusion

The analysis of pollen DNA from samples of different ages collected from different sites makes it possible to directly assess the history and dynamics of ancient plant taxa and to understand the microevolutionary processes that occurred during the recent past. In particular, the combination of ancient sequences and coalescent methods is a powerful tool to reconstruct detailed demographic histories, test models of population genetics, and understand the interplay between microevolution and environment. For example, by analyzing DNA extracted from ancient pollen, one should be able to investigate how ancient populations responded to specific climate changes in the past and to answer specific questions, such as these: When exactly did tree species lose or gain genetic diversity during the postglacial population migration from the southern refugia? or, Is there any correlation between changes in genetic diversity and climate events that occurred during postglacial migration?

Because pollen is probably the most widely distributed Holocene fossil and is available in large numbers, further developments in the extraction and analysis of DNA from fossil material will provide answers to the questions of plant movement in space and time by reducing the taxonomic resolution from species or generic level to population or species level. Although the study of plant aDNA is still in its youth as compared to that of animals, the availability of suitable material for the former has been demonstrated. The timescale for aDNA analysis covers the last glacial–interglacial transition, and for a considerable portion of this period, accurate and precise chronological data can be obtained by using radiocarbon dating methods. Additionally, several studies on the phylogeography of modern plants, especially trees (Lascoux et al. 2004), provide adequate baseline data for comparison with aDNA results. Therefore, it is possible to create a dated record of changes in phylogeography in time as well as space.

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